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A DNA minor groove binder shows high effectiveness as a quencher for FRET probes

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ABSTRACT

A non-fluorescent quencher based on thiazole orange was incorporated into oligonucleotides. Fluorimetry and fluorogenic real-time polymerase chain reaction experiments demonstrated that the quencher is effective for fluorescein amidite dyes. The thiazole orange quencher also increased the melting temperature of DNA duplexes, which may facilitate the design of shorter and more discriminatory probes. The effectiveness of the quencher in TaqMan probes was also demonstrated.

Fluorescence quenchers are employed in a wide variety of fluorometric assays for various purposes such as the detection of nucleic acids,[1–3] assessment of enzymatic activity,[4–6] and detection of other molecules of interest with turn-on responses.[7,8] Nucleic acid hybridization assays represent an important class of techniques in modern biology, and such assays have diverse applications including the diagnosis of inherited diseases, determination of human identity, identification of microorganisms, paternity testing, evaluation of virology, and DNA sequencing (i.e., sequencing by hybridization). A particularly important class of methods used in nucleic acid hybridization assays employs a reporter-quencher energytransfer dye pair comprising a reporter dye and a quencher dye that interact through fluorescence resonance energy transfer (FRET). Typically, an oligonucleotide probe is doubly labeled with a fluorophore and a proximal quencher molecule, and hybridization of the probe to the target nucleic acid leads to signal generation via a conformational change in the probe. Examples include Scorpion primers and molecular beacons. TaqMan probes have a different mode of action that relies upon enzymatic cleavage to separate the fluorophore and quencher. However, certain limitations plague many of the dye/quencher pairs commonly used in fluorogenic primers and probes. These include insufficient spectral overlap between the dye and quencher and/or intrinsic fluorescence of the quencher, both of which can reduce the fluorescent signal-to-noise ratio. As examples, the nonfluorescentdiazo-dyes DABCYL[9] and methyl red,[10] which are frequently used in real-time polymerase chain reaction (PCR) probes, do not efficiently quench the fluorescence of several commonly used "long wavelength" fluorophores, such as the cyanine dye Cy[5], that are widely used in fluorescent probes and are particularly important in DNA microarrays. Therefore, a new range of quenchers have been developed based on azo dyes [e.g., Black Hole Quencher (BHQ) 1, 2, and 3, BiosearchTechnologies; Eclipse Dark Quencher, Epoch Biosciences, Inc.; BlackBerry Quencher; and other azo dyes commonly used as fluorescent dyes].

Some small molecules can react with DNA via covalent or noncovalent interactions, and noncovalent interactions are of greatest interest for use in dye/quencher pairs. There are several sites in the DNA molecule where noncovalent binding can occur, such as: (i) between two base pairs (complete intercalation), (ii) in the minor groove, (iii) in the major groove, and (iv) on the outside of the helix.[11] Lerman[12] was the first to propose that planar organic compounds can bind to DNA via intercalation, and since that groundbreaking work, much research has investigated the binding of small molecules to DNA.[13] One such small molecule, thiazole orange, exhibits a low level of autofluorescence in free form but becomes highly fluorescent upon intercalation into DNA.[14]

Recently, we developed a new fluorescence quencher based on thiazole orange. This quencher is non-fluorescent, thereby eliminating unwanted background signals that can arise with the use of fluorophores as quenchers. Importantly, this quencher has a absorption peak in the 528 nm region of the visible spectrum

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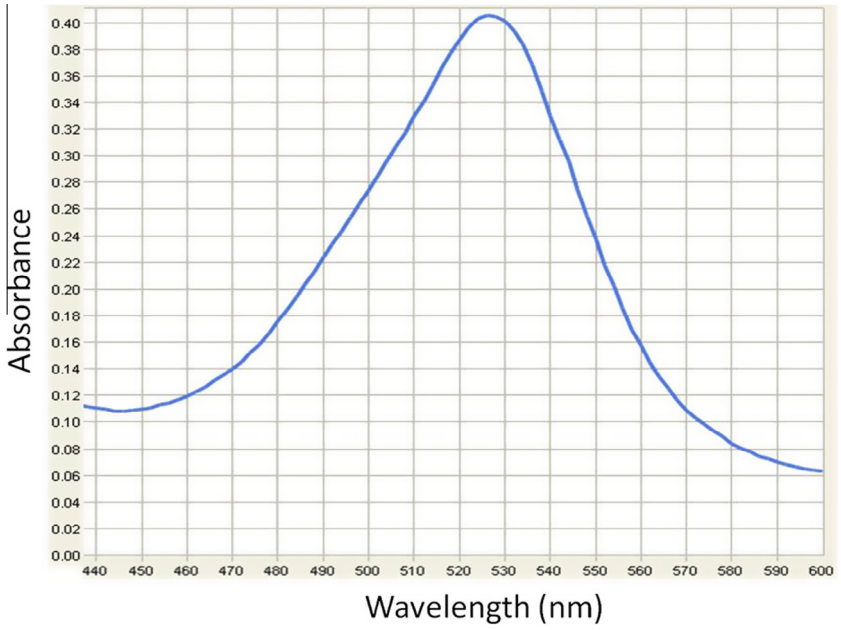

Figure 1. Absorption spectrum of the thiazole orange.

(Fig. 1) and is thus able to quench fluorescein amidite (FAM). Here, we report the synthesis of a form of this quencher that is suitable for incorporation into oligonucleotide probes at either the 3′ or 5′ end or internally in place of a thymidine. We also determined the degree of fluorescence quenching with each placement. In addition, experiments were performed to demonstrate the utility of the new quencher in TaqMan probes for real-time PCR, as well as the effect of the quencher on duplex stability.

Briefly, the non-fluorescent thiazole orange quencher is prepared as follows (Fig. 2): A quaternizedbenzazole derivative is mixed with lepidinium salts[15] and refluxed under basic conditions (e.g., diisopropylethylamine in methanol or pyridine). The solvent is evaporated and the remaining solid is washed with dilute hydrochloric acid (e.g., 5% HCl in water) and dried. The dyes are rendered amino-reactive via the conversion of a carboxylic acid group to a succinimidyl ester by dissolving the dye in dimethylformamide (DMF) with succinimidyltetramethyluronium salt and *N*,*N*-diisopropylethylamine (DIPEA). The product is precipitated by the addition of dilute HCl, washed, and dried.

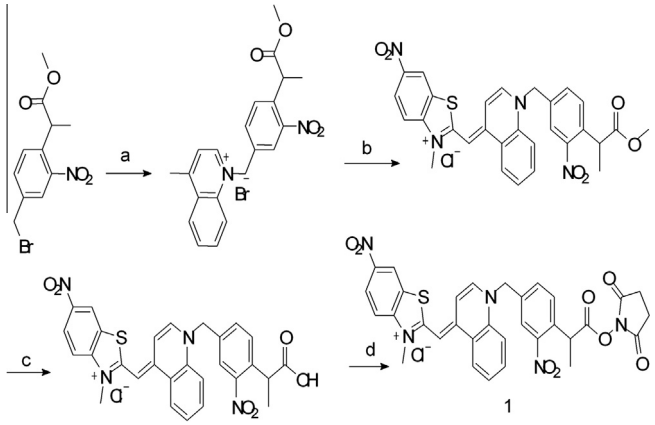

Figure 2. (a) Lepidine, 110 °C, overnight; (b) 2-(ethylthio)-6-nitrobenzothiazole, MeOH, and DIPEA, room temperature, overnight; (c) MeOH/H_2O = 1:1 and NaOH, room temperature, overnight and (d) DMF, DIPEA, and TSTU. Room temperature, 2 h. [*O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoroborate.]

The FRET quenching efficiency of the thiazole orange (TO) quencher was determined via TaqMan PCR and compared to that of BHQ-1. To prepare a functional probe, the fluorophore FAM was attached to the 5′ end of the sequence 5′-TGCAGTCCTCGCTCACTGGGCAC-3′, and the quencher (i.e., none, TO, or BHQ-1) was attached to the 3′ end of the sequence. Guided by the sequences on WHO website (http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/), we designed three probes for determining the quenching efficiency as follows (InfA = universal influenza A):

InfA-forward primer:	5′-GACCRATCYTGTCACCTCTGAC-3′
InfA-reverse primer:	5′-AGGGCATTYTGGACAAAKCGTCTA-3′
InfA-probe 1:	5′-FAM-TGCAGTCCTCGCTCACTGGGCAC-3′
InfA-probe 2:	5′-FAM-TGCAGTCCTCGCTCACTGGGCAC-TO-3′
InfA-probe 3:	5′-FAM-TGCAGTCCTCGCTCACTGGGCAC-BHQ1-3′

As shown in Figure 3a, because the infA-P^1 probe did not contain a quencher, the fluorescence of FAM was not quenched. Thus, the background was high, and no signal was detected upon amplification. By contrast, the infA-P^2 and infA-P^3 probes contained the TO quencher and BHQ-1, respectively, and both quenched the fluorescence of FAM. The fluorescent background at the start of the reaction was low, and in line with amplification of the PCR product, the fluorescence of FAM increased slowly as enzymatic cleavage progressed. There was a significant amplification curve, and the background generated by the TO quencher was lower than that generated by BHQ-1. Additionally, a oligonucleotide with a different sequence (forward: CCCACCRAGCAACAAACG, reverse: CCTTCCGACATCAGCTTCACT) was detected by the way mentioned above. The same result was found to be in consistence with the phenomenon that TO quencher was more effective than BHQ-1 as shown in Figure 3b.

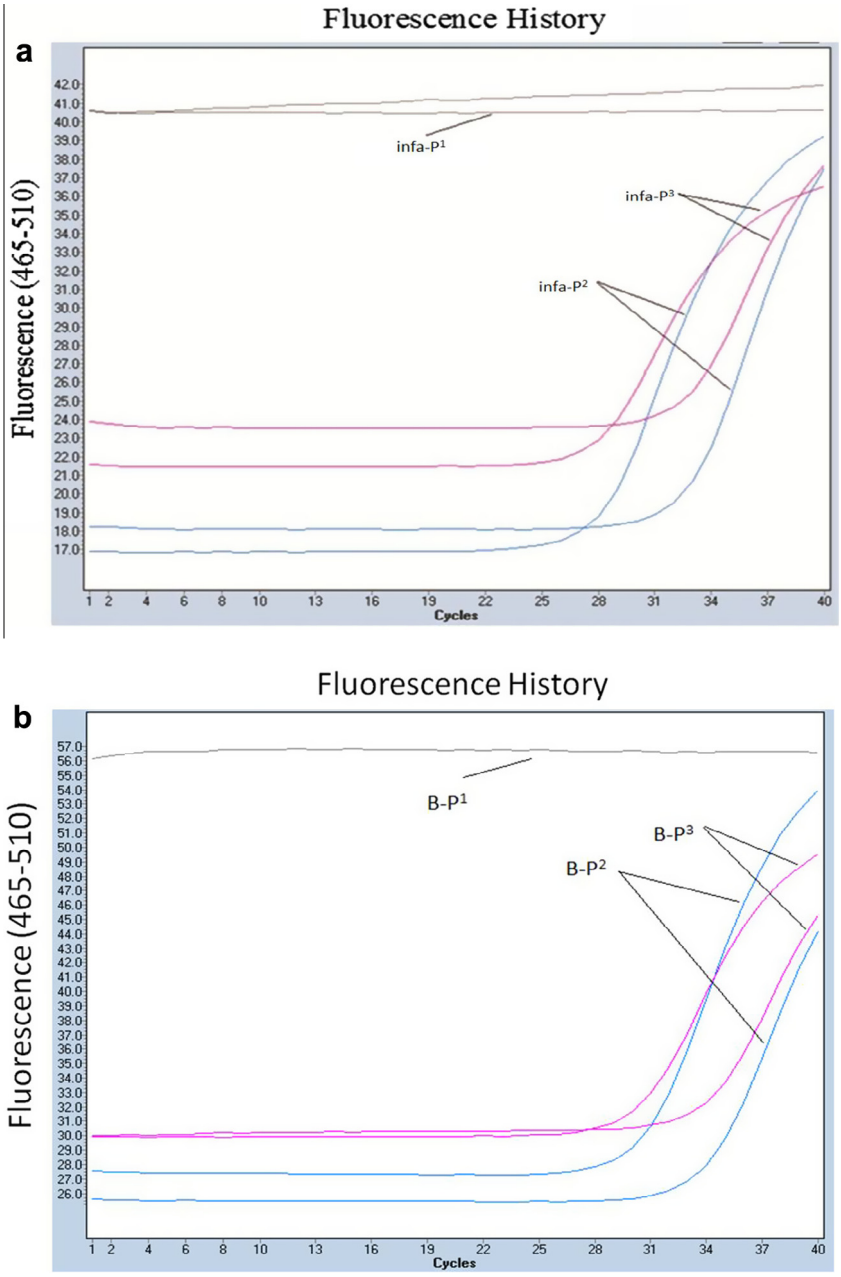

Figure 3. (a,b) FRET quenching efficiencies of the TO quencher and BHQ-1. 3a: Compound of the sequence of 5′-TGCAGTCCTCGCTCACTGGGCAC-3′. 3b: Compound of the sequence of 5′ CCCGGAACCCATCCCCGGA-3′. Note: infA-P^1 or B-P^1: probe without a quencher; infA-P^2 or B-P^2: probes contained the TO quencher; infA-P^3 or B-P^3: probes contained the BHQ-1 quencher. Blue or red identical color curves: different concentration of same probe. Vertical axis: Absolute value of fluorescence.

A TaqMan probe for *Mycobacterium tuberculosis* that has a melting temperature (T_m) of 58 °C was synthesized via labeling with FAM and BHQ-1, and another probe was prepared via labeling with FAM and the TO quencher. Probes with the following sequences were prepared:

Forward primer:	5′-CCTGACTCGTCAACTTCACG-3′
Reverse primer:	5′-GCGTGTACACCACCACTCC-3′
Probe 1:	5′-FAM- AAGCGCCGAGTTCGG-BHQ1-3′
Probe 2:	5′-FAM- AAGCGCCGAGTTCGG-TO-3′

As shown in Figure 4, because BHQ-1 could not increase the T_m, the T_m of Probe 1 remained at 58 °C, which was lower than the annealing temperature (60 °C) in the PCR amplification process. Thus, the probe and template were not effectively combined, as demonstrated by both hysteresis of the C_t value of the amplification curve and a reduced increase in fluorescence. By contrast, because the TO quencher increased the T_m of the DNA duplexes to a value greater than the annealing temperature of the amplification, a normal amplification curve was achieved with Probe 2, which contained this quencher. The data in Figure 4 also demonstrate that using the TaqMan method, fluorescence quenching occurs via FRET[16] within the intact probe.

To examine the influence of TO on the melting temperature, a melting analysis was done on the mixture of quencher labeled oligonucleotides with a fluorophore labeled complementary oligonucleotide. Data revealed that the T_m was increased from 52 to 59 centigrade by TO as shown in Figure 5.

In conclusion, a non-fluorescent quencher based on TO was incorporated into synthetic oligonucleotides at the 3′ ends. The quencher is able to quench fluorophores that emit in the

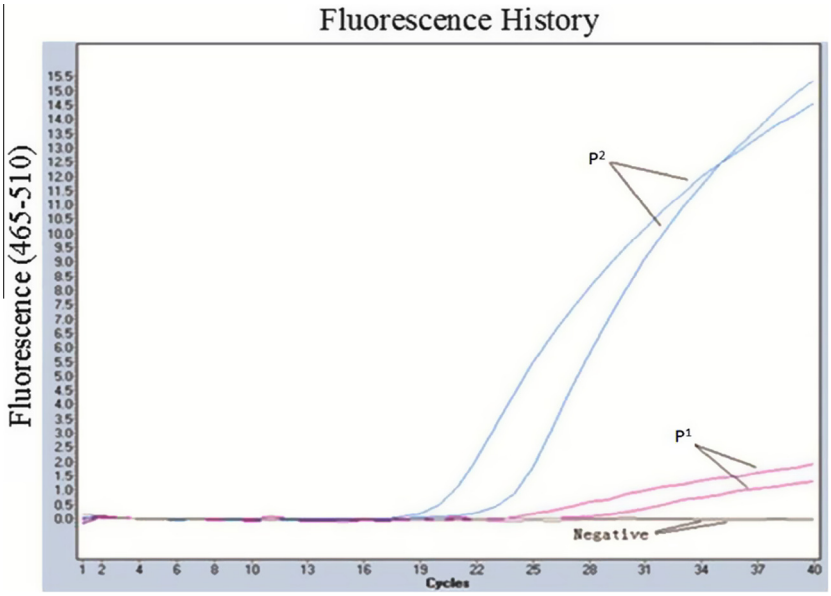

Figure 4. DNA stability using the TO quencher versus BHQ-1. *Note:* Negative: water; P1: probes contained the BHQ-1 quencher; P2: probes contained the TO quencher. Blue or red identical color curves: different concentration of same probe. Vertical axis: Increased value of fluorescence.

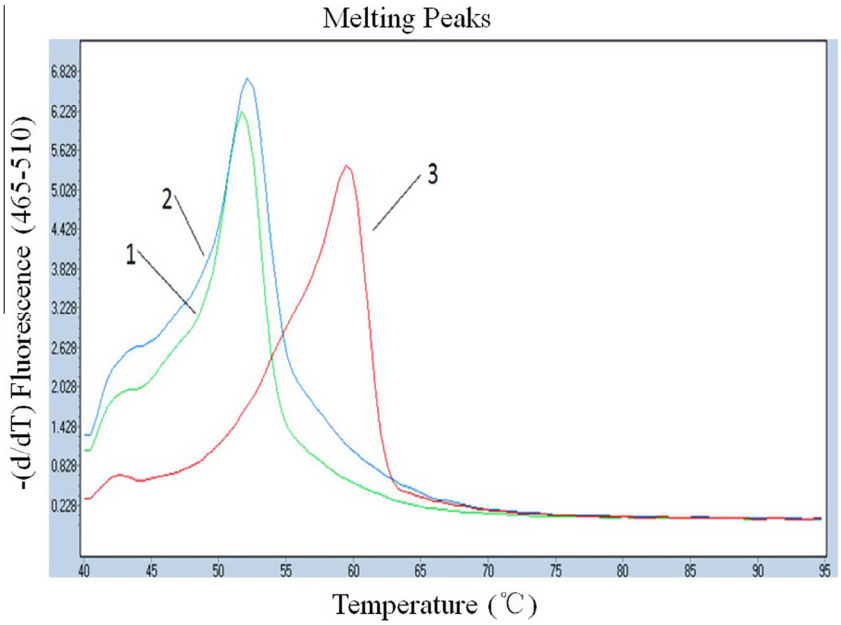

Figure 5. Influence of TO on the melting temperature. *Note:* '1': Melting curve of the double strand by two no-labeled complementary oligonucleotides. Its T_m was 52 °C. '2': Melting curve of the double strand by one oligonucleotide labeled with BHQ1 and one no-labeled complementary oligonucleotides. Its T_m was 52.5 °C. Obviously, BHQ1 could hardly change the temperature. '3': Melting curve of the double strand by one oligonucleotide labeled with TO and one no-labeled complementary oligonucleotides. Its T_m was 59 °C. It revealed that TO could increase the temperature effectively.

520–540 nm range. The utility of the quencher was demonstrated in real-time PCR using TaqMan probes. As shown in Figure 3, the signal-to-noise ratios obtained with the TO quencher were improved compared to those observed for BHQ-1 when quenching FAM fluorescence. Because the TO quencher can significantly stabilize DNA duplexes by increasing their melting temperature, use of this quencher may facilitate the design of shorter, more specific probes.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.06.036.

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